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ABSTRACT

Psychotic symptoms are a common and debilitating feature of Alzheimer's disease (AD) and are associated with a more rapid course of decline. Current evidence from postmortem and neuroimaging studies implicates frontal, temporal, and parietal lobes, with reported disruptions in monoaminergic pathways. However, the molecular mechanisms underlying this remain unclear. In the present study, we investigated methylomic variation associated with AD psychosis in 3 key brain regions implicated in the etiology of psychosis (prefrontal cortex, entorhinal cortex, and superior temporal gyrus) in postmortem brain samples from 29 AD donors with psychosis and 18 matched AD donors without psychosis. We identified psychosis-associated methylomic changes in a number of loci, with these genes being enriched in known schizophrenia-associated genetic and epigenetic variants. One of these known loci resided in the *AS3MT* gene—previously implicated in schizophrenia in a large GWAS meta-analysis. We used bisulfite-pyrosequencing to confirm hypomethylation across 4 neighboring CpG sites in the *ASM3T* gene. Finally, our regional analysis nominated multiple CpG sites in *TBX15* and *WT1*, which are genes that have been previously implicated in AD. Thus one potential implication from our study is whether psychosis-associated variation drives reported associations in AD case-control studies.

1. Introduction

Around 40% of people with Alzheimer's disease (AD) will at some point experience psychotic symptoms, which are distressing, have a major negative impact on disease course and accelerate the need for nursing home care (Connors et al., 2018) and for which there are no effective licensed treatments (Creese et al., 2018). The limited knowledge of disease mechanisms underlying psychosis in AD (AD + P) represents a major obstacle in the identification of novel treatment targets and understanding the syndrome at a clinical level. Neuropathological studies implicate increased pTau and TDP-43 pathology in the pathogenesis of AD + P (Murray et al., 2014). The heritability of AD + P is estimated to be 61% (Bacanu et al., 2005) and linkage studies (Hollingworth et al., 2007) and population level analyses of common single-nucleotide polymorphisms and copy number variants provide further support for a genetic basis to the syndrome (Barral et al., 2015; Hollingworth et al., 2007; Zheng et al., 2015). Interestingly, some of these studies suggest genetic links with schizophrenia (SZ) (Creese et al., 2019; DeMichele-Sweet et al., 2018), thus raising the question as to whether there are common mechanisms that underpin psychosis across the life span. These genomic and neuropathological studies provide compelling evidence that AD + P represent a syndrome of AD with a distinct neurobiological profile, potentially offering exciting opportunities for precision medicine. However, neuropathology explains only ~18% of AD + P variance (Krivinko et al., 2018), whereas the genomics of other complex disorders suggest that common single-nucleotide polymorphisms alone are likely to only explain a small proportion of variance (Golan et al., 2014). Therefore, further research is required to provide a better understanding of the molecular mechanisms

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underlying AD + P, and whether this presents novel precision treatment opportunities.

In recent years, epigenome-wide association studies (EWAS) have transformed our understanding of the molecular etiology of AD (Lunnon et al., 2014; Smith et al., 2019, 2018) and psychiatric conditions, including SZ (Viana et al., 2017). Here, we present the first EWAS of AD + P in cortical samples from pathologically confirmed AD cases that had undergone a standardized assessment of psychotic symptoms during life, hypothesizing that alterations in DNA methylation are associated with AD + P.

2. Materials and methods

2.1. Sample selection

This study used DNA methylomic data previously generated by our group using postmortem human brain tissue from the MRC London Neurodegenerative Disease Brain Bank using the Illumina Infinium HumanMethylation450 K BeadChip (Lunnon et al., 2014) (GEO accession number GSE59685). Data from a total of 141 matched cortical samples from the entorhinal cortex (EC), prefrontal cortex (PFC), and superior temporal gyrus (STG) were used from 29 AD + P subjects and 18 AD subjects without psychosis (AD-P) (Supplementary Table 1). Samples and clinical data were collected as part of the Alzheimer's Research UK funded study "Biomarkers of AD Neurodegeneration"; participants were recruited through secondary care in England with informed consent provided according to the Declaration of Helsinki (World Medical Association, 1991). During life, all cases received a clinical diagnosis of dementia. At postmortem, all cases underwent a thorough standardized examination and all cases in the present analysis were diagnosed with AD postmortem. There was no significant difference in the distribution of neurofibrillary tangle Braak stage between the AD-P and AD + P groups. The following comorbid pathologies were present in the cohort but numbers (provided in Supplementary Table 1) were not significantly different across AD + P and AD-P groups: TDP-43, cerebral amyloid angiopathy (CAA), vascular pathology (including small vessel disease), Lewy body pathology (brain stem or limbic predominant). There were no cases with cortical Lewy body disease. Psychosis was assessed using the Neuropsychiatric Inventory (NPI), a ten-item scale measuring a range of neuropsychiatric symptoms reported in dementia (Cummings et al., 1994). Assessment with NPI was undertaken at baseline and average Mini-Mental State Examination at the time of assessment was 12.7 (SD = 9). For this analysis, symptoms were coded as present (>0 on either the delusion or hallucination items of the NPI) or absent (no symptoms).

2.2. Illumina 450K array data analysis

The raw signal intensities for the arrays were imported into R (version 3.6). Stringent quality control and normalization were performed separately for each region using wateRmelon and methylumi packages in R as described previously (Lunnon et al., 2014). The minfi package in R was used to estimate the proportion of neuronal cells across samples for each tissue (Aryee et al., 2014). The effects of age, sex, and derived neuronal cell proportion were regressed out from the normalized methylation beta values for all samples before subsequent analysis. Principal component analysis demonstrated that variables such as postmortem interval and batch did not impact on the data and so these were not included as covariates. Global analysis of DNA methylation data were estimated by the cumulative distribution function of the methylation values for each individual using B-spline basis functions using the GAMP package in R (v.0.11) (Zhao et al., 2015). The estimated global DNA methylation levels in the AD + P and AD-P groups were compared in each of the cortical brain regions. To identify differentially methylated positions (DMPs) consistently associated with AD + P across all 3 cortical regions, we performed a linear model analysis using generalized least squares ('gls' function in nlme R package [Pinheiro et al., 2019]), which allows for a fully unstructured variance-covariance matrix of the residuals. Fixed effects were specified in such a way that we directly obtained the pooled estimate and standard error across the brain regions. Quantile-quantile (Q-Q) plots were used to assess the inflation index (Supplementary Fig 1). DMPs were ranked by both p-value and the magnitude of effect size. To identify differentially methylated regions (DMRs), we used the Python module comb-p to group $\geq$3 spatially correlated p-values in a 500-bp sliding window (Pedersen et al., 2012). To test for an enrichment of the AD + P EWAS loci in known SZ GWAS variants we used Fisher's method to combine together AD + P EWAS p-values for probes residing in the independent genomewide significantly associated regions nominated in the most recent SZ GWAS meta-analysis (Pardinas et al., 2018). Of the 145 regions identified by Pardinas et al, 101 contained >1 CpG site on the 450K array and were used in our analyses. Finally, to test for an enrichment of the AD + P EWAS loci in known SZ EWAS variants, we used a one-sided Fisher's test to test whether the 1000 top-ranked AD + P-associated probes in our EWAS were enriched in a list of 1894 significant SZ-associated probes ($P_{FDR} < 0.05$) from a recent SZ EWAS of PFC (Jaffe et al., 2016).

2.3. Targeted validation using bisulfite pyrosequencing

Bisulfite pyrosequencing was used to replicate DNA methylation in the same samples and tissues across 6 individual CpG sites in the *AS3MT* gene, spanning from chr10:104,629,829-104,629,929 (hg19). Bisulfite conversion was performed using the EZ DNA Methylation-Gold kit (Zymo Research, USA). A single amplicon (101 bp) was generated using primers designed using the PyroMark Assay Design software 2.0 (Qiagen, UK). Pyrosequencing was performed using 2 sequencing primers to maximize coverage across the 5 CpG sites. DNA methylation was quantified using the Pyromark Q24 system (Qiagen, UK) using the manufacturer's standard instructions and the Pyro Q24 CpG 2.0.6 software. To examine the combined effect of the CpGs across the designed amplicon for pyrosequencing, the measured DNA methylation for each site was tested using the generalized least square method and estimates and corresponding variance-covariance matrix of the estimates were subjected to the "rma" function in the metafor R package (v2.1) (Viechtbauer, 2010).

3. Results and discussion

3.1. AD + P is characterized by common patterns of methylation across brain regions

The aim of this study was to identify distinct patterns of DNA methylation associated with the presence of psychosis in AD. Therefore, we performed an EWAS in the 29 AD + P and 18 AD-P samples, while controlling for confounders such as age, sex, and derived neuronal cell proportions. First, we used the cumulative distribution function of the methylation values for each individual to quantify global methylation levels across samples and observed no difference in global DNA methylation between AD + P and AD-P individuals in any of the 3 brain regions examined (PFC: $p = 0.99$, EC: $p = 0.52$, STG: $p = 0.76$). Next, we examined DNA methylation differences between AD + P and AD-P subjects at individual loci covered by the array, with the 1000 top-ranked loci (based on both effect size and p value) shown in Supplementary Table 2. Although no CpGs survived the stringent Bonferroni significance threshold of $p < 1.66E-07$, a number of loci showed similar patterns of

methylation across all 3 brain regions. Interestingly, the top-ranked probe (cg19596870, estimate = −0.173, $p = 3.42E-04$) and the 11th-ranked probe (cg01266060, estimate = −0.029, $p = 3.00E-05$) reside within the *SERPINB6* gene, located 498 bp downstream from the TSS and in the gene body, respectively. This gene is expressed in the brain and has been previously identified in an SZ-coagulation gene interaction network (Huang et al., 2014). We also identified DMPs annotated to other genes that have previously been implicated in SZ; our 76th ranked loci resided within the body of the *AS3MT* gene (cg08772003, estimate = −0.025, $p = 7.50E-04$), where increased gene expression has been linked to SZ risk alleles in the 10q24.32 SZ-related locus.

3.2. A number of DMRs spanning multiple adjacent CpGs are seen in AD + P

We identified 2 DMRs, each with consistent hypomethylation in the AD + P group in all 3 cortical regions (Supplementary Table 3). These regions corresponded to 10 CpG sites (613 bp) within the first exon of the *TBX15* gene (Fig. 1A: $P_{Sidak} = 5.88E-09$) and 8 CpG sites (476 bp) in the first intron of the *WT1* gene (Fig. 1B: $P_{Sidak} = 3.00E-08$).

3.3. Differentially methylated loci in AD + P are enriched for known SZ variants

Given that several of our top-ranked DMPs appeared to have been previously associated with SZ, we were interested whether there was a significant enrichment of significant DMPs within known SZ-associated variants. To this end, using the most recent list of independent SZ-associated genomic regions from Pardinas et al., (2018), we examined the enrichment of AD + P-associated DMPs residing in the linkage disequilibrium blocks harboring risk variants. 101 of the 145 linkage disequilibrium blocks contained >1 CpG site on the 450K array and using Fisher's method we combined *p*-values within each of these blocks, identifying a significant enrichment in our data in locus 1 after correcting for multiple testing (Chr6: 24,988,105-33,842,877; xMHC (10,409 probes), $P_{FDR} = 1.21E-04$) (Supplementary Table 4). Notably, we observed that 40 of the genes annotated to our 1000 top ranked AD + P probes were in the significant SZ EWAS probe list with the same direction of effect, including *TBX15*. When we correlated the t-statistics of our EWAS with the SZ EWAS for these 40 CpG sites we observed a highly significant correlation ($r = 0.87$, $p = 2.672E-13$). Finally, we found 19 of the genes we identified in our AD + P EWAS were present in the list of 376 differentially expressed genes (at FDR) in the recent meta-analysis of transcriptomic data in SZ by Manchia et al., (2017), demonstrating a significant enrichment ($p = 0.035$).

3.4. Bisulfite pyrosequencing replicates hypomethylation of the AS3MT gene in cortex

We used bisulfite pyrosequencing to quantify DNA methylation across an extended region of 101 bp spanning 5 CpG sites, including cg08772003, one of the top-ranked DMPs we had identified within the exonic region of *AS3MT*. This gene was selected for validation given its reported role in the pathogenesis of SZ (Li et al., 2016). In our 450K analysis, we had observed hypomethylation in AD + P at cg08772003 in all 3 cortical regions (Fig. 2A). Using pyrosequencing, we observed a trend toward significant hypomethylation across the 3 regions (Fig. 2B), with DNA methylation values calculated on the 450K array being highly correlated with the values calculated by pyrosequencing (Fig. 2C). Our pyrosequencing assay covered 4 additional CpG sites, 3 of which were downstream of cg08772003 (Supplementary Table 5). Interestingly, these 3 CpGs all showed the same direction of effect as the CpG covered by the 450K probe (hypomethylation) (Fig. 2D–E), and when we averaged methylation across the 4 hypomethylated sites, these showed significant hypomethylation ($p = 0.0011$) associated with AD + P.

4. Conclusions

We examined cross-cortical DNA methylation changes associated with AD + P in a series of clinically and neuropsychological well-characterized cases. We found consistent patterns of DNA

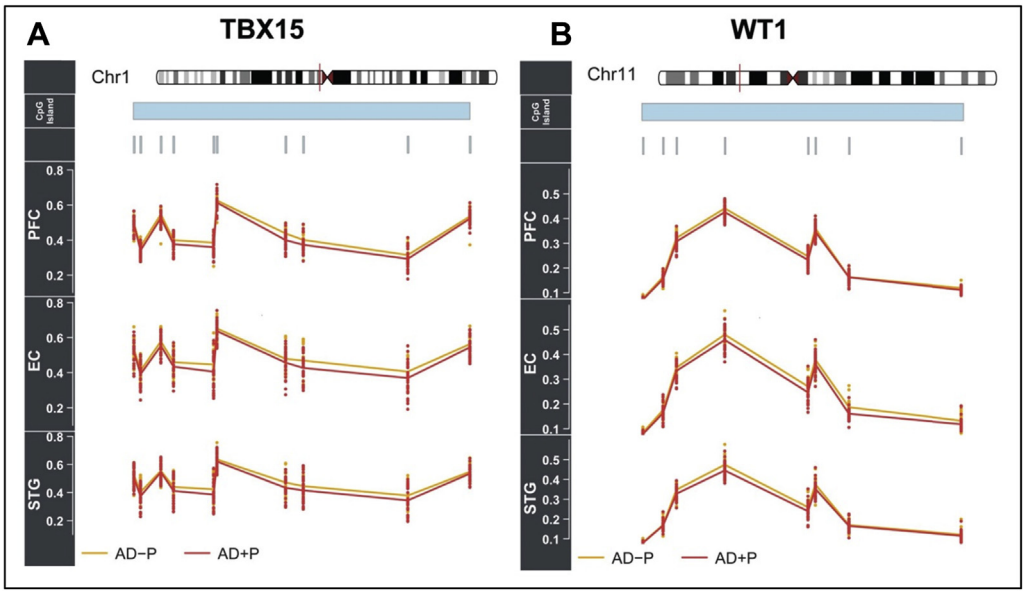

Fig. 1. Two DMRs consisting of multiple adjacent DMPs can be identified in AD + P. (A) Ten DMPs showed hypomethylation in *TBX15* and (B) 8 DMPs in *WT1* showed hypomethylation in AD + P (red) compared with AD-P (orange) in all 3 cortical brain regions. Shown on the X axis is genomic location. Shown on the Y axis is the corrected DNA methylation level (%). Abbreviations: AD, Alzheimer's disease; AD + P, psychosis in AD; DMR, differentially methylated region. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

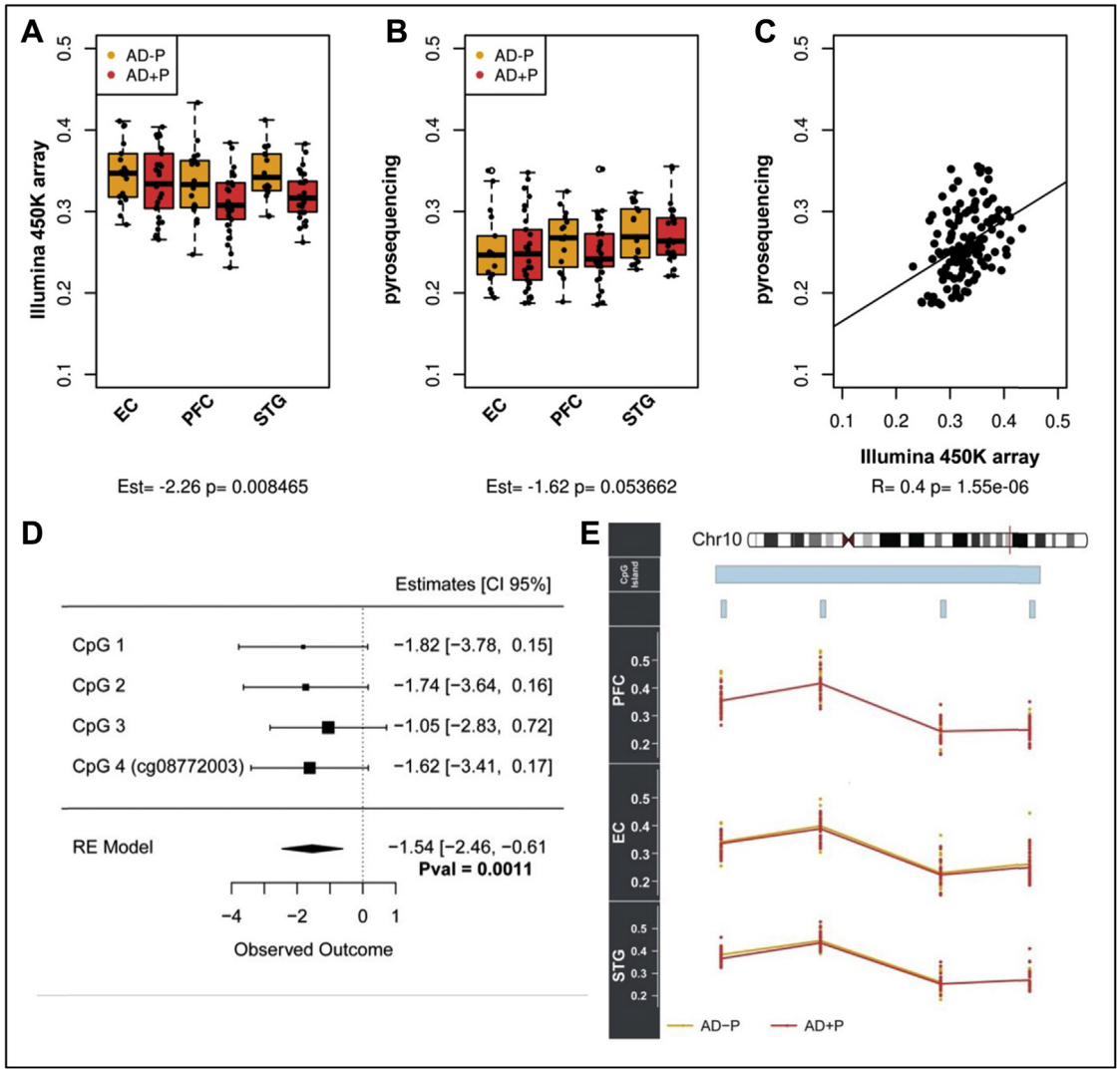

Fig. 2. *AS3MT* shows consistent hypomethylation in AD + P using 2 different technologies. In the 450K array data, we demonstrated significant AD + P-associated hypomethylation across all 3 cortical brain regions at cg08772003 (A), with a similar trend at this loci when replicated using pyrosequencing (B) and a significant correlation of methylation levels calculated by the 2 technologies (C). Three neighboring downstream CpG sites covered by the pyrosequencing assay also showed psychosis-associated hypomethylation across the cortex, which was significant across the region (D, E). Abbreviations: AD, Alzheimer's disease; AD + P, psychosis in AD; DMR, differentially methylated region.

methylation across entorhinal, temporal, and frontal cortex, with the top-ranked loci being enriched for known EWAS and GWAS SZ loci. To our knowledge, this is the first evidence implicating DNA methylation in AD + P and adds further support for transdiagnostic hypotheses linking psychotic disorders across the life span.

We identified DMRs in *TBX15* and *WT1*, which were hypomethylated in AD + P relative to AD-P. It is interesting to note that these genes have been previously reported in the context of AD; *TBX15* has been shown to be hypomethylated in the STG of AD cases relatives to non-AD controls (Watson et al., 2016), while *WT1* has been previously shown to be present in neurofibrillary tangles (Lovell et al., 2003). Although our findings should be replicated in independent cohorts, one potential important broader implication from our study is whether AD + P-associated variation drives reported genetic, epigenetic, or transcriptomic associations previously identified between AD cases and non-AD controls. Given that around ~40% of patients with AD will experience psychosis (Connors et al., 2018), which is seldom measured or reported, it is plausible that psychosis could represent a significant confounder in AD case-control studies, which cannot easily be accounted for.

There are several converging lines of evidence to suggest that psychotic symptoms across the life span have some common mechanisms. Recent genomic research has linked polygenic risk for SZ to psychotic symptoms in Huntington's disease (Ellis et al., 2019) and AD (Creese et al., 2019), as well as psychotic experiences in the general population (Legge et al., 2019), while neuropsychological testing implicates similar deficits in processing speed and executive function in individuals with very-late-onset SZ-like psychosis and AD + P (Van Assche et al., 2019). Our findings that top-ranked AD + P-associated DMPs are enriched for SZ loci extend this evidence base into molecular-level mechanistic similarities for the first time.

We are unable at present to determine whether the AD + P-associated DNA methylation patterns we have identified are causal or a consequence of the psychotic episodes. Nonetheless, given that many of the top ranked loci overlapped with known psychosis-associated genetic variants, it does suggest that some of the epigenetic variation we have identified may lie upstream of symptom onset. There are a number of other limitations to our study, for example the use of bulk tissue, which contains both glia and neuronal cell types and our relatively small sample size in this

study. Although we were able to validate AD + P-associated hypomethylation in the *AS3MT* gene using another technology, this was on the same samples and thus it will be important in the future to validate our findings in additional similarly well-characterized cohorts. Nonetheless, the quality of clinical and neuropathological phenotyping is a key strength of our study and our findings provide a clear rationale for further molecular level profiling of AD + P.

Disclosure statement

All the authors declare that they have no conflicts of interest with this work.

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This study used epigenome-wide association study (EWAS) Illumina 450K DNA methylation data that had already been generated in human postmortem brain tissue from donors in the MRC London Neurodegenerative Disease Brain Bank and these data are available on GEO. In the course of this study, the authors also generated pyrosequencing data using the DNA that had been isolated for that EWAS. Ethical approval for our project was awarded by the University of Exeter Medical School research ethics committee.

Authors' contributions: EP contributed to conceptualization, methodology, software, writing—original draft, writing—review and editing preparation, formal analysis, and investigation. BC contributed to conceptualization, writing—original draft preparation, writing—review and editing, formal analysis, and investigation. ARS contributed to investigation and validation. WV contributed to methodology. PP contributed to resources. DLAvdH contributed to funding acquisition and supervision. CB contributed to conceptualization and resources. JM contributed to funding acquisition, supervision, writing—review and editing, and resources. KL contributed to conceptualization, writing—original draft, writing—review and editing, supervision, project administration, and funding acquisition.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neurobiolaging.2020.01.001.

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